J. Biochem. 124, 1077-1085 (1998)

Purification and Characterization of a Serine Protease in Erythrocyte Cytosol That Is Adherent to Oxidized Membranes and Preferentially Degrades Proteins Modified by Oxidation and Glycation[1]

Tomofumi Fujino, Tomohiro Tada, Masatoshi Beppu, and Kiyomi Kikugawa[2]

School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392

Received for publication, May 6, 1998

A serine protease that preferentially degrades oxidized and glycated proteins was shown to be present in erythrocyte cytosol. Human erythrocyte cytosol was labeled with [^{3}H]diisopropyl fluorophosphate (DFP) and passed through a column of carboxymethyl-Sephadex to obtain radioactive fractions free of hemoglobin. The fractions contained a single radioactive 80-kDa protein, as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE)/fluorography. The radioactive 80-kDa protein bound to unoxidized erythrocyte membranes, and more effectively to oxidized membranes. The radioactive protein was purified through a column of diethylaminoethyl-cellulose and by preparative native-PAGE in a purity of 80%. Antibody against the cytosolic 80-kDa protein bound to 80-kDa protein of erythrocyte membranes, indicating the presence of the same protein in the membrane. The antibody bound more effectively to oxidized membranes than to unoxidized membranes. The 80-kDa protein partially purified from unlabeled cytosol degraded more effectively oxidized bovine serum albumin (BSA), oxidized IgG, and glycated BSA more effectively than the corresponding unoxidized or unglycated proteins. Degradation of oxidized or glycated proteins was effectively inhibited by DFP. Hence, the protein is an 80-kDa serine protease that is adherent to oxidized membranes and is responsible for degradation of proteins modified by oxidation and glycation.

Key words: diisopropyl fluorophosphate, erythrocyte serine protease, glycation, oxidation, protein degradation.

Proteases that preferentially degrade oxidatively damaged proteins participate in what is referred to as the secondary antioxidant defense system (*1*). In erythrocytes, a multicatalytic proteolytic complex composed of multiple subunits has been shown to be responsible for the degradation of oxidized intracellular proteins (*2-4*). Our previous studies have shown that oxidative stress renders erythrocyte membrane proteins susceptible to degradation by membrane-bound serine protease (*5*), and an 80-kDa serine protease loosely bound to the oxidized membranes may be responsible for the degradation of oxidized membrane proteins (*6*). It has been suggested that the same 80-kDa serine protease detected in oxidized erythrocyte membranes is present in cytosol, and the cytosolic protease is readily bound to membranes when the cells are oxidized (*6*).

The aim of the present study was to purify and characterize the serine protease in erythrocyte cytosol and elucidate its selectivity for proteins modified by oxidation or glycation. It was found that the 80-kDa serine protease in cytosol becomes adherent to membranes upon cell oxidation and has high selectivity for proteins modified by oxidation and glycation.

MATERIALS AND METHODS

Materials—Xanthine (X), xanthine oxidase (XO) [EC 1.1.3.22] (from buttermilk, grade III), diisopropyl fluorophosphate (DFP), bovine serum albumin (BSA) (fatty acid and γ-globulin free), horseradish peroxidase (HRP) [EC 1.11.1.7], trypsin [EC 3.4.21.4] [TPCK-treated], α-chymotrypsin [EC 3.4.21.1], and Nonidet P-40 were obtained from Sigma Chemical (St. Louis, MO, USA). XO was treated before use as described (*7*). [1,3-^{3}H]DFP ([^{3}H]DFP) (6.0 Ci/mmol) and ^{125}I-Bolton-Hunter reagent (*N*-succinimidyl-3-(4-hydroxy-3-[^{125}I]iodophenyl)propionate) (2,335 Ci/mmol) were obtained from NEN Research Products (Boston, MA, USA).

Human venous blood withdrawn from a healthy donor using acid-citrate-dextrose as an anticoagulant was stored

[1] This work was supported in part by a Grant for private universities provided by Japan Private School Promotion Foundation, and by a Grant-in-Aid for Scientific Research (No. 09672256) from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Tel: +81-426-76-4503, Fax: +81-426-76-4508

Abbreviations: BSA, bovine serum albumin; CM, carboxymethyl; DEAE, diethylaminoethyl; DFP, diisopropyl fluorophosphate; DPBS (+), Dulbecco's phosphate-buffered saline; DPBS(−), Ca^{2+}-, Mg^{2+}-free DPBS; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; X, xanthine; XO, xanthine oxidase.

© 1998 by The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

at 4°C and used within one week. Blood was centrifuged ($320\times g$, 10 min) at 4°C to remove plasma and buffy coats. Erythrocyte pellet was washed four times by centrifugation ($320\times g$, 10 min) at 4°C with ice-cold Ca^{2+}-, Mg^{2+}-free Dulbecco's phosphate-buffered saline (pH 7.4) [DPBS (−)]. This procedure yielded an erythrocyte suspension free of leukocytes (*5*). Human IgG was purified according to the method previously described (*7*).

Analysis—Proteins in the membrane preparations were determined by the method of Lowry *et al.* (*8*) using BSA as a reference standard. Hemoglobin content in erythrocyte cytosol was determined by absorbance at 523 nm and its molar extinction coefficient of 7,880 (*7*). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) or native-PAGE was performed according to the method of Laemmli (*9*) using 25 mM Tris/192 mM glycine (pH 8.3) with or without 0.1% SDS in a discontinuous buffer system with a 7.5 or 10% separating gel and a 4% stacking gel under reducing conditions at 10 mA for 2.5 h unless otherwise mentioned. Protein bands in the gel were stained with Coomassie Brilliant Blue R-250 (CBB) or silver (Ag) by use of a staining kit (Dai-ichi Chemicals, Tokyo). For fluorographic detection of ^3H radioactivity, the PAGE gel was treated with EN^{3}HANCE (NEN Research Products) according to the manufacturer's instructions, dried, and the radioactivity was visualized by use of a Kodak XAR-5 X-ray film with the aid of an enhancing screen at −80°C.

Oxidation of Erythrocytes—Erythrocytes were oxidized with X/XO/Fe(III) at 1 mM/10 mU ml^{-1}/0.1 mM in Dulbecco's phosphate buffered saline (pH 7.4) [DPBS (+)] at 37°C for 3 h as described previously (*5*). Unoxidized erythrocytes were obtained under the same conditions without the oxidants.

Preparation of Erythrocyte Membranes and Cytosol—Erythrocyte pellets from oxidized and unoxidized erythrocytes were lysed in 40 volumes of 5 mM sodium phosphate buffer (pH 8.0) containing 0.1 mM α-tocopherol. Each lysate was centrifuged at $9,600\times g$ for 20 min to obtain an erythrocyte membrane pellet. The membrane pellet was washed several times with the same buffer by the same centrifugation until the supernatant became colorless. The membrane pellet was resuspended in DPBS (+). All the operations were performed at 4°C. The membrane suspensions were stored at −80°C until use.

Erythrocyte cytosol was prepared as previously reported (*10*). An erythrocyte pellet was lysed in 1.5 volume of 1 mM dithiothreitol for 1 h. The mixture was centrifuged at $10,000\times g$ for 20 min to obtain the supernatant, which was then centrifuged at $18,500\times g$ for 60 min. The supernatant was dialyzed against a solution of 10 mM Tris-HCl buffer (pH 7.8)/5 mM $MgCl_2$/0.5 mM dithiothreitol/8 mM KCl/10% glycerol to obtain erythrocyte cytosol. The cytosol preparation was stored at −80°C until use.

[^3H]DFP-Labeling of Erythrocyte Membranes and Partial Purification of 80-kDa Protein—Erythrocyte membranes were labeled with [^{3}H]DFP, and the labeled 80-kDa protein was partially purified according to the method described (*6*). In brief, erythrocyte membrane suspension (5 mg protein) was labeled with [^{3}H]DFP, and the 0.1% Nonidet P-40-soluble fraction was separated through a column of Sepharose CL-6B to obtain the second radioactive fraction F II as erythrocyte membrane 80-kDa serine protease (9.6×10^4 cpm/ml).

[^3H]DFP-Labeling of Erythrocyte Cytosol for Purification of 80-kDa Protein and for Measurement of Proteolytic Activity—For purification of 80-kDa protein in erythrocyte cytosol, [^{3}H]DFP-labeled cytosol was used. Erythrocyte cytosol (100 ml, 9 g hemoglobin) was treated with 2 mM DFP at 4°C for 15 h, then dialyzed against 10 mM sodium phosphate buffer (pH 7.0)/1 mM EDTA to prepare DFP-modified cytosol. Separately, 20 μl of a solution of 1 mCi/ml (0.1 μmol/ml) [^{3}H]DFP was added to 2 ml of erythrocyte cytosol (180 mg hemoglobin), and the mixture was stirred at room temperature for 3 h. To inactivate remaining DFP-reactive serine residues, unlabeled DFP was added to the mixture at the final concentration of 2 mM, and the mixture was kept at 4°C for 15 h. The mixture was dialyzed against 10 mM sodium phosphate buffer (pH 7.0)/1 mM EDTA. Specific activity of [^{3}H]DFP-labeled erythrocyte cytosol was 6,300 cpm/mg hemoglobin. [^{3}H]DFP-labeled erythrocyte cytosol (2 ml, 1.1×10^6 cpm, 180 mg hemoglobin) was diluted with DFP-modified erythrocyte cytosol (100 ml, 9 g hemoglobin) to reduce the specific radioactivity to 1/50 to prepare [^{3}H]DFP-labeled cytosol (102 ml, 9.18 g hemoglobin, 1.1×10^6 cpm) for purification of [^{3}H]DFP-labeled protein.

For measurement of proteolytic activity of the 80-kDa protein in cytosol, a small amount of [^{3}H]DFP-labeled cytosol was added to untreated cytosol as a purification marker. Untreated cytosol (100 ml, 9 g hemoglobin) was mixed with [^{3}H]DFP-labeled cytosol (2 ml, 180 mg hemoglobin, 1.1×10^6 cpm) to obtain native/[^{3}H]DFP-labeled cytosol (102 ml, 9.18 g hemoglobin, 1.1×10^6 cpm).

Carboxymethyl (CM)-Sephadex Ion Exchange Column Chromatography of [^3H]DFP-Labeled Erythrocyte Cytosol—[^{3}H]DFP-labeled cytosol (102 ml, 9.18 g hemoglobin, 1.1×10^6 cpm) was applied to a column (5.2 cm i.d. × 96 cm) of CM-Sephadex (Pharmacia Biotech, Uppsala, Sweden). The column was eluted with 4,200 ml of 10 mM sodium phosphate buffer/1 mM EDTA (pH 7.0) and subsequently with 3,600 ml of 10 mM sodium phosphate buffer/1 mM EDTA/0.5 M NaCl (pH 9.2), and radioactivity and absorbance at 577 (for hemoglobin) and 280 nm (for protein) of each fraction were measured. Radioactive fractions (CM fractions, 200 ml) were obtained in the void volume.

Diethylaminoethyl (DEAE)-Cellulose Ion Exchange Column Chromatography of Radioactive CM Fractions—CM fractions (200 ml) were applied to a column (2.6 cm i.d. × 40 cm) of DEAE-cellulose (DE-52, Whatman International, Maidstone, England), and the column was eluted with 400 ml of 10 mM sodium phosphate buffer (pH 7.0)/1 mM EDTA in a linear gradient fashion with NaCl between 0 and 0.8 M. Radioactivity and absorbance at 577 and 280 nm of each fraction were measured. The radioactive fractions obtained (DEAE fractions, 250 ml) were dialyzed against 10 mM sodium phosphate buffer (pH 7.0)/1 mM EDTA, and applied to a small column (0.9 cm i.d. × 8 cm) of DEAE-cellulose. The column was washed with 10 ml of the same buffer, then radioactivity was eluted with the same buffer containing 0.5 M NaCl. The radioactive fraction (6 ml) was concentrated to 1 ml in a dialysis bag by aspiration, then dialyzed against 62.5 mM Tris-HCl (pH 6.8)/0.1 mM EDTA/10% glycerol.

Preparative Native-PAGE of Radioactive DEAE Fractions—Preparative native-PAGE was performed using an

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

Atto biophoresis III AE 6700C machine (Tokyo). The concentrated DEAE fractions (1 ml) were treated with dithiothreitol (1.6 mg/ml) at 37°C for 3 h, then applied to a column gel (1.6 cm i.d. × 10 cm) composed of 4.5% stacking gel and 7.5% separating gel. Electrophoresis was performed in 25 mM Tris/192 mM glycine (pH 8.3) in a discontinuous buffer system at 15 mA and 15°C for 8.5 h, and electrophoretically separated proteins were collected in fractions of 0.8 ml using 372 mM Tris-HCl (pH 8.8)/10% glycerol as a carrier solution at a flow rate of 0.8 ml/min. Bromophenol blue was used as a top marker of the electrophoresis. Radioactivity and absorbance at 280 nm of each fraction were measured. The radioactive fractions obtained (native-PAGE fractions, 3 ml) were dialyzed against 10 mM sodium phosphate buffer (pH 7.0)/1 mM EDTA at 4°C.

Preparation of Antibody against DFP-Reactive Cytosolic 80-kDa Protein (Anti Cytosolic 80-kDa Protein)—Antibody against DFP-reactive 80-kDa protein was obtained by immunization of a rabbit with the mixture of the native PAGE fractions of erythrocyte cytosol containing 400 μg of 80-kDa protein and Freund's complete ajduvant (Difco Laboratories, Detroit, MI, USA). IgG was separated from the antiserum by ammonium sulfate precipitation and DEAE ion exchange chromatography, and the specific antibody was further purified by affinity chromatography as follows. Native PAGE fractions were subjected to SDS-PAGE using a slab gel, and the band of 80-kDa protein, which was identified by brief staining with CBB, was cut from the gel with a razor blade. The gel strip taken was homogenized in 0.1 M sodium bicarbonate (pH 8.3)/0.5% SDS, and the gel homogenate was centrifuged to obtain protein extract in the supernatant. The 80-kDa protein thus purified was coupled to a HiTrap NHS-activated Sepharose column (Pharmacia Biotech) according to the manufacturer's instructions. To this 80-kDa protein-coupled column, the IgG fraction from the antiserum was applied, and the antibody adsorbed to the column was eluted with 0.1 M glycine-HCl (pH 2.6). After neutralization with 1 M Tris-HCl (pH 7.0), the eluate was dialyzed against DPBS (−)/0.02% sodium azide and used as an affinity-purified antibody against cytosolic 80-kDa protein (anti cytosolic 80-kDa protein). The concentration of the antibody was determined using E (1%, 1 cm) 280 nm = 13.5 (*11*).

Detection of Membrane-Bound 80-kDa Protein by Immunoblotting and Enzyme-Linked Immunosorbent Assay (ELISA)—Immunoblotting detection of 80-kDa protein of erythrocyte membranes was performed according to the method of Towbin *et al.* (*12*) with modifications. Briefly, erythrocyte membranes were solubilized in Laemmli's sample buffer and subjected to SDS-PAGE. Protein bands on the gel were transferred onto a PVDF membrane, and the membrane was coated with 2% BSA in 10 mM Tris-HCl buffer (pH 7.4)/0.15 M NaCl (Tris-buffered saline; TBS). The membrane was successively incubated with affinity-purified anti cytosolic 80-kDa protein (2.5 μg/ml) and ^{125}I-labeled goat anti rabbit IgG (500,000 cpm/ml, ICN Biochemicals, Costa Mesa, CA, USA). The radioactive bands on the membrane was visualized by radioluminography using a Fujix BAS 2000 Bio-Imaging Analyzer (Fuji, Tokyo).

Antigen activity of 80-kDa protein in erythrocyte membranes was measured by competitive inhibition of ELISA as follows. An aliquot (100 μl) of DEAE fractions of 80-kDa protein diluted with TBS (0.25 μg of 80-kDa protein/ml) was placed in each well of a 96-well plastic plate and evaporated to dryness at 45°C. The wells were washed four times with TBS/0.01% Tween 20 and coated with 2% BSA in TBS. A solution of anti cytosolic 80-kDa protein diluted with TBS to 0.25 μg/ml was incubated at 4°C for 1 h with an equal volume of a solution of erythrocyte membranes solubilized in 0.2% Triton X-100/DPBS(−), and 100 μl of the mixture was placed in the 80-kDa protein-coated wells. After 1 h of incubation, the wells were washed four times with TBS/0.01% Tween 20. The antibody bound to the wells was measured by incubation with a protein A-horseradish peroxidase conjugate (Bio-Rad Laboratories, Hercules, CA, USA) followed by measurement of the bound peroxidase activity using *o*-phenylenediamine and H_2O_2. The brown color developed was measured by absorbance at 492 nm. The absorbance of a similarly processed control well, a well not coated with DEAE fractions of 80-kDa protein, was subtracted as a blank.

Preparation of Oxidized and Unoxidized BSA and IgG, and ^{125}I-Radiolabeling—A mixture of 10 ml of a solution of BSA or IgG at 0.5 mg/ml in 50 mM Tris-HCl (pH 7.8), 1.0 ml of 0.2 M H_2O_2 in the same buffer, and 0.1 ml of a solution of HRP at 0.1 mg/ml in the same buffer was incubated at 37°C for 20 h. The mixture was then dialyzed against 10 mM sodium phosphate buffer (pH 8.0) to obtain oxidized BSA or IgG. BSA or IgG was similarly treated without the oxidant to obtain unoxidized BSA or IgG.

A 200-μl solution of 100 μg of protein was mixed with 250 μCi ^{125}I-Bolton-Hunter reagent (*13*), and the mixture was stirred at 4°C for 15 h. To stop the reaction, 50 μl of 50 mM phosphate buffer (pH 7.5) containing 10 mg/ml glycine was added, and the mixture was stirred at 4°C for 1 h. The mixture was then applied to a column of Sephadex G-50 precoated with 1% gelatin, and the column was eluted with 10 mM sodium phosphate buffer (pH 7.4)/0.02% sodium azide to obtain ^{125}I-labeled protein. Specific radioactivity of ^{125}I-oxidized BSA, unoxidized BSA, oxidized IgG, and unoxidized IgG was 1.4×10^6, 2.8×10^6, 3.8×10^6, and 4.3×10^6 cpm/μg protein, respectively. The ^{125}I-oxidized and unoxidized preparations were diluted with unlabeled oxidized and unoxidized preparations, respectively, to reduce their specific radioactivity to 1/10.

Preparation of Glycated BSA and ^{125}I-Radiolabeling—A solution of 160 mg of BSA and 300 mg of glucose in 1.0 ml of 0.5 M sodium phosphate buffer (pH 7.4) was sterilized by passing through a Millipore filter (0.2 μm) and incubated at 37°C for 14 days in a sealed tube. After dilution with 10 mM sodium phosphate buffer (pH 7.4)/0.02% sodium azide, the mixture was dialyzed against the same buffer. BSA was similarly treated without glucose to obtain non-glycated BSA. The amount of free amino groups in the glycated BSA was determined using fluorescamine. To a solution of 10 μg of glycated BSA in 750 μl of 50 mM sodium phosphate buffer (pH 8.0) was added 250 μl of a solution of 75 μg of fluorescamine in dioxane under stirring for 5 min. Relative fluorescence intensity of the solution at 390 (excitation)/475 nm (emission) was measured and compared with that obtained from 10 μg of BSA to assess the amount of residual amino groups in glycated BSA. Only 12% free amino groups remained in glycated BSA. Glycated

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

and non-glycated BSA were labeled with ^{125}I as described above. Specific radioactivity of ^{125}I-glycated and non-glycated BSA was 1.9×10^6 and 2.1×10^6 cpm/μg protein, respectively. The ^{125}I-glycated and non-glycated BSA were diluted with unlabeled glycated and non-glycated BSA, respectively, to reduce their specific radioactivity to 1/10.

Degradation of ^{125}I-Oxidized and Glycated Proteins—A mixture of 100 μl of a solution of the 80-kDa protein preparation (4 or 9.5 μg protein), trypsin (1 μg) or α-chymotrypsin (1 μg) in 10 mM sodium phosphate buffer (pH 8.0)/1 mM EDTA/0.02% sodium azide, and 5 μl of a solution of ^{125}I-oxidized or glycated protein preparation (1 μg protein) in 10 mM sodium phosphate buffer (pH 8.0) was incubated at 37°C for the indicated period. For investigation of the effect of DFP, 1 μl of a solution of 0.2 M DFP was added to 100 μl of the enzyme solution. To the mixture was added 395 μl of a solution of BSA at 1 mg/ml as a carrier protein, then 500 μl of cooled 20% trichloroacetic acid, and the mixture was cooled on ice for 1 h. Radioactivity of the whole mixture was counted. After centrifugation at 8,000$\times g$ for 10 min, trichloroacetic acid-soluble radioactivity in 500 μl of the supernatant was counted. Radioactivities without added enzyme solution were subtracted.

RESULTS

The present study examined whether the 80-kDa serine protease found in oxidized erythrocyte membranes (*6*) is present in erythrocyte cytosol. All operations for purification of the enzyme were performed in the presence of EDTA in order to block the activities of any contaminating metalloproteases. Erythrocyte cytosol was labeled with [^{3}H]DFP, and the radioactive cytosol was passed through a column of CM-Sephadex to remove hemoglobin. Radioactivity was recovered in the void volume of the column (CM fractions) in a recovery of 64%, and hemoglobin was retained in the column. SDS- and native-PAGE under reducing conditions and subsequent fluorography of radioactive CM fractions (Fig. 1, lanes left b and right b) showed that the fractions contained a single radioactive 80-kDa protein. The electrophoretic position of the protein band coincided with that of 80-kDa serine protease obtained by [^{3}H]DFP labeling and subsequent partial purification of erythrocyte membranes (Fig. 1, lanes left a and right a), suggesting that the membrane 80-kDa serine protease is present in cytosol. Binding activity of the cytosolic 80-kDa protein in CM fractions to unoxidized and oxidized erythrocyte membranes was measured (Table I). Radioactivity of CM fractions bound to membranes obtained from unoxidized erythrocytes, and much more effectively to membranes obtained from erythrocytes gently oxidized with X/XO/Fe(III). Thus, the 80-kDa protein in erythrocyte cytosol was much more adherent to oxidized membranes than to unoxidized membranes.

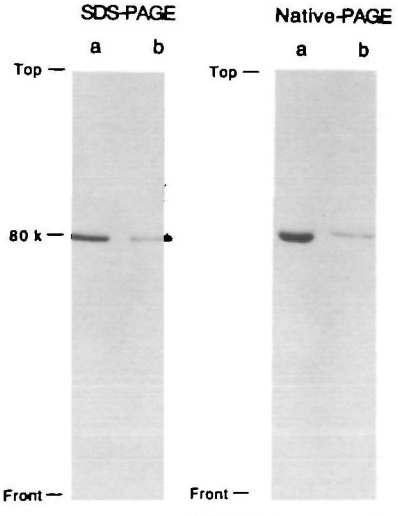

Fig. 1. **SDS-PAGE and native-PAGE/fluorography using 7.5% separating gel of partially purified [^{3}H]DFP-labeled erythrocyte membrane 80-kDa serine protease (a) and radioactive CM fractions (b).** Radioactivity loaded: lane a, 2,000 cpm; lane b, 1,280 cpm.

The 80-kDa protein was purified further using the protein whose active center serine was fully labeled with [^{3}H]DFP. DEAE-cellulose ion exchange column chromatography of radioactive CM fractions showed major radioactive fractions as a single peak separated from a large amount of other cytosolic proteins (Fig. 2A). These radioactive fractions (DEAE fractions) were analyzed on SDS- and native-PAGE under reducing conditions (Fig. 2B). SDS-PAGE showed that the fractions were composed of 80-kDa protein and other proteins when detected by CBB and Ag staining. Native-PAGE showed that the fractions were composed of 80-kDa protein and a large amount of other proteins migrating to the top of the gel. SDS-PAGE of DEAE fractions under nonreducing conditions and subsequent fluorography showed no radioactive bands of higher molecular weight (data not shown), indicating that the protein was not cross-linked to other proteins by S-S bonds.

The 80-kDa protein in radioactive DEAE fractions was further purified by preparative native-PAGE. A single radioactive peak was obtained in fractions around fraction number 80. These radioactive fractions (native-PAGE fractions) were analyzed by gel electrophoresis (Fig. 3). On native-PAGE, the [^{3}H]DFP-labeled 80-kDa protein was detected as a single band by Ag staining. SDS-PAGE showed that native-PAGE fractions were little contaminated by other proteins detectable by Ag and CBB staining. Densitometric determination of CBB-stained 80-kDa protein (1.6×10^4 cpm) on SDS-PAGE revealed that 81% of the density was associated with the 80-kDa protein. Hence, the purity of 80-kDa serine protease thus purified was estimated to be about 80%. The amount of the 80-kDa protein on the CBB-stained gel was estimated to be 8 μg on

TABLE I. **Binding of radioactive CM fractions to unoxidized and oxidized erythrocyte membranes.** A mixture of 1 ml of radioactive CM fractions diluted with DPBS (+) at 5×10^4 cpm/ml and 1 ml of unoxidized or oxidized erythrocyte membrane suspension in DPBS (+) was incubated at 37°C for 3 h with gentle shaking. The mixture was filtered through a glass microfiber filter GF/C (Whatman International, Maidstone, England), and the filter was washed twice with 5 ml of ice-cold DPBS (+), then dried at 50°C for 20 h in a vial. Radioactivity on the filter was then counted using a toluene scintillator. Radioactivity in the absence of membranes was subtracted. The data are the means±SD of triplicate experiments.

	Radioactivity retained on the filter (cpm)
Unoxidized erythrocyte membranes	72±16
Oxidized erythrocyte membranes	990±92

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

the basis of a calibration curve obtained by the densitometric determination of the CBB-stained SDS-PAGE gel of a reference standard BSA (0–20 μg). Hence, the specific activity of the protein was found to be 2×10^3 cpm/μg. The amount of the radioactive 80-kDa serine protease obtained from erythrocyte cytosol originating from 100 ml erythrocytes was estimated to be 700 μg. The overall recovery of the 80-kDa protein was estimated to be 33% by assuming that all the radioactivity of the starting cytosol was due to [^{3}H]DFP-labeled 80-kDa protein. The recovery at each step of the purification is summarized in Table II.

To confirm the identity of the DFP-reactive 80-kDa protein in cytosol with that in membranes, antibody against the cytosolic 80-kDa protein was prepared by immunizing a rabbit with native-PAGE fractions and by isolating a specific antibody by affinity chromatography using a column of purified cytosolic 80-kDa protein that was extracted from a SDS-PAGE gel of native-PAGE fractions. The affinity-purified antibody bound only to 80-kDa protein on a Western blot of whole erythrocyte proteins, while IgG from an unimmunized rabbit did not bind at all, indicating that the antibody specifically binds to 80-kDa protein (data not shown). Unoxidized and oxidized erythrocyte membranes were subjected to SDS-PAGE followed by immunoblotting. As shown in Fig. 4, the antibody bound to 80-kDa protein of unoxidized and oxidized erythrocyte membranes, indicating that 80-kDa protein in cytosol is identical to that in membrane. The extent of the antibody binding to 80-kDa protein of oxidized erythrocyte membranes was greater than that to 80-kDa protein of unoxidized erythrocyte membranes. This indicates that a greater amount of 80-kDa protein is present in oxidized membranes than in unoxidized membranes. Consistent with these results, unoxidized and oxidized membranes exhibited the 80-kDa protein antigen activity in competitive inhibition of ELISA, and the antigen activity of oxidized membranes was more potent than that of unoxidized membranes (Fig. 5). Based on a standard inhibition curve obtained by the use of

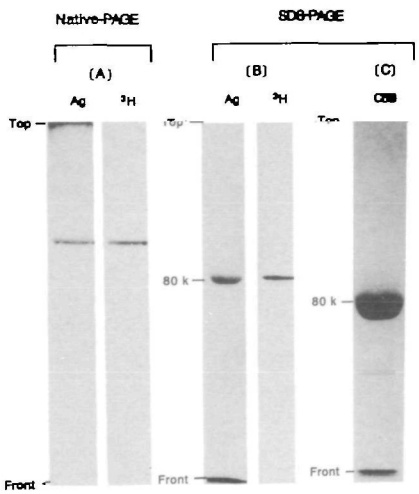

Fig. 3. **Native-PAGE and SDS-PAGE (7.5% separating gel)/CBB staining, Ag staining, and fluorography of radioactive native-PAGE fractions.** The sample (1,200 cpm/lane) was loaded onto native-PAGE (A) and SDS-PAGE (B) and proteins were detected by Ag staining and fluorography. The sample (1.6×10^3 cpm/lane) was loaded onto SDS-PAGE (C) and proteins were detected by CBB staining.

TABLE II. **Recovery of DFP-reactive 80-kDa protein of erythrocyte cytosol in the purification steps.** The yield of the DFP-reactive 80-kDa protein at each step of its purification was calculated using specific radioactivity of 2,000 cpm/μg protein, which was obtained as described in the text, and assuming that the radioactivity was entirely due to [^{3}H]DFP-labeled 80-kDa protein.

Purification step	Radioactivity of [^{3}H]DFP-labeled protein (cpm/100 ml erythrocytes)	DFP-reactive 80-kDa protein	
		Yield (g/100 ml erythrocytes)	Recovery (%)
Cytosol	4.2×10^6	2.14	(100)
CM fractions	2.7×10^6	1.36	(64)
DEAE fractions	2.3×10^6	1.17	(55)
Native-PAGE fractions	1.4×10^6	0.70	(33)

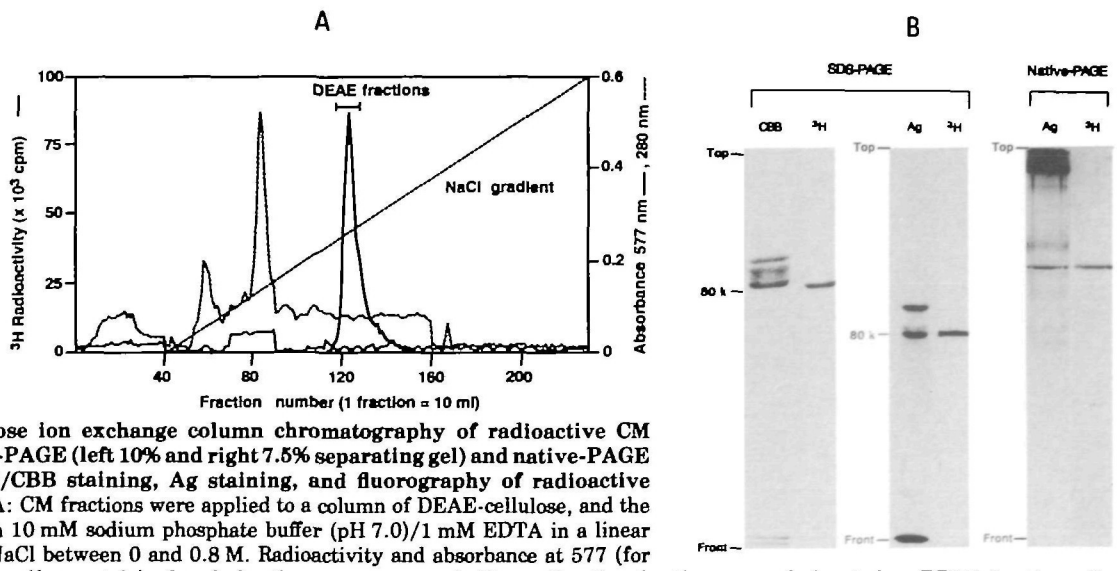

Fig. 2. **DEAE-cellulose ion exchange column chromatography of radioactive CM fractions (A) and SDS-PAGE (left 10% and right 7.5% separating gel) and native-PAGE (7.5% separating gel)/CBB staining, Ag staining, and fluorography of radioactive DEAE fractions (B).** A: CM fractions were applied to a column of DEAE-cellulose, and the column was eluted with 10 mM sodium phosphate buffer (pH 7.0)/1 mM EDTA in a linear gradient fashion with NaCl between 0 and 0.8 M. Radioactivity and absorbance at 577 (for hemoglobin) and 280 nm (for protein) of each fraction were measured. The radioactive fractions were designated as DEAE fractions. B: Radioactivity loaded on each lane: 6,280 cpm.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

cytosolic 80-kDa protein, the content of 80-kDa protein of unoxidized and oxidized membranes was estimated to be 180 and 410 μg/g protein, respectively.

In order to characterize the proteolytic activity of the 80-kDa protein, the native protein in cytosol, with an added small amount of [^{3}H]DFP-labeled cytosol as a marker, was purified according to the method described above. Untreated cytosol (100 ml, 9 g hemoglobin) was mixed with [^{3}H]-DFP-labeled cytosol (2 ml, 180 mg hemoglobin, 1.1×10^6 cpm) to prepare a mixture of native/[^{3}H]DFP-labeled cytosol. Native/[^{3}H]DFP-labeled cytosol was passed successively through columns of CM-Sephadex and DEAE-cellulose and finally subjected to preparative native-PAGE to obtain the purified fractions containing unlabeled and [^{3}H]DFP-labeled 80-kDa protein. After purification by DEAE-cellulose and preparative native-PAGE, radioactive fractions with about 6×10^5 cpm and 3.5×10^5 cpm were obtained, respectively.

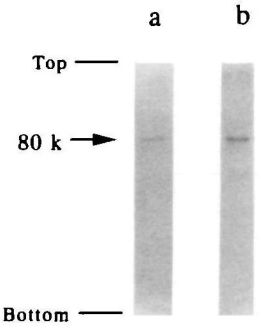

Fig. 4. **Immunoreactivity of 80-kDa protein of unoxidized (a) and oxidized (b) erythrocyte membranes with anti cytosolic 80-kDa protein as detected by immunoblotting.** Membranes from unoxidized (a) and oxidized (b) erythrocytes were subjected to SDS-PAGE, followed by immunoblotting detection of the membrane protein that is reactive with anti cytosolic 80-kDa protein using ^{125}I-labeled goat anti rabbit IgG as a secondary antibody. Protein of the erythrocyte membranes loaded on each lane: 23 μg.

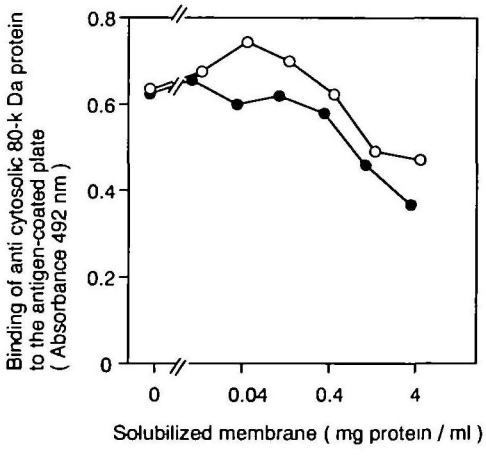

Fig. 5. **Immunoreactivity of unoxidized and oxidized erythrocyte membrane protein with anti cytosolic 80-kDa protein as detected by competitive inhibition of ELISA.** Membranes from unoxidized (○) and oxidized (●) erythrocytes were solubilized with 0.2% Triton X-100/DPBS(−), and their inhibitory activity against the binding of anti cytosolic 80-kDa protein to the antigen-coated wells in ELISA was measured at the concentrations indicated.

Proteolytic activity of the purified protein against oxidized BSA, oxidized IgG, and glycated BSA was examined. Oxidized BSA and IgG preparations were obtained by oxidation of BSA and human IgG with H_2O_2/HRP at 20 mM/1 μg·ml^{-1} at 37°C for 20 h, and a glycated BSA preparation was obtained by incubation of BSA with glucose at 37°C for 14 days. The modified and unmodified proteins were radiolabeled with ^{125}I. The mixture of the purified 80-kDa protein fractions and each of ^{125}I-labeled modified and corresponding unmodified proteins was incubated at 37°C, and the proteolytic activity was assessed by the release of trichloroacetic acid-soluble ^{125}I radioactivity. Time courses of protein degradation by DEAE fractions of ^{125}I-labeled unoxidized BSA and oxidized BSA (Fig. 6A), ^{125}I-labeled unoxidized IgG and oxidized IgG (Fig. 6B), and ^{125}I-labeled non-glycated BSA and glycated BSA (Fig. 6C) showed that the oxidized or glycated proteins were more

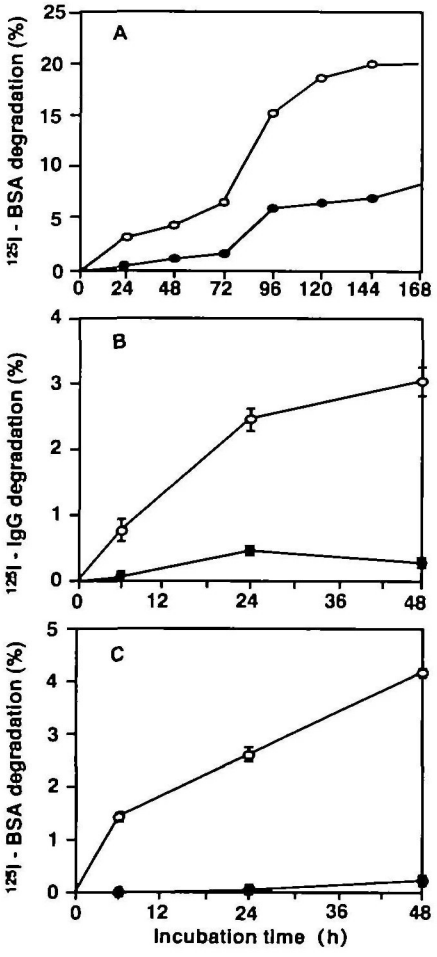

Fig. 6. **Time course of the degradation of ^{125}I-unoxidized and oxidized BSA (A), ^{125}I-unoxidized and oxidized IgG (B), and ^{125}I-nonglycated and glycated BSA (C) by 80-kDa protein (DEAE fractions) from native/[^{3}H]DFP-labeled cytosol.** A mixture of (A) ^{125}I-unoxidized (●) and oxidized BSA (1 μg) (○), (B) ^{125}I-unoxidized (●) and oxidized IgG (1 μg) (○), or (C) ^{125}I-nonglycated (●) and glycated BSA (1 μg) (○), and DEAE fractions (4 μg protein) in 105 μl of 10 mM sodium phosphate buffer (pH 8.0)/1 mM EDTA was incubated at 37°C for the indicated period. Protein degradation was examined by the release of trichloroacetic acid-soluble radioactivity. The data are the means of triplicate experiments.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

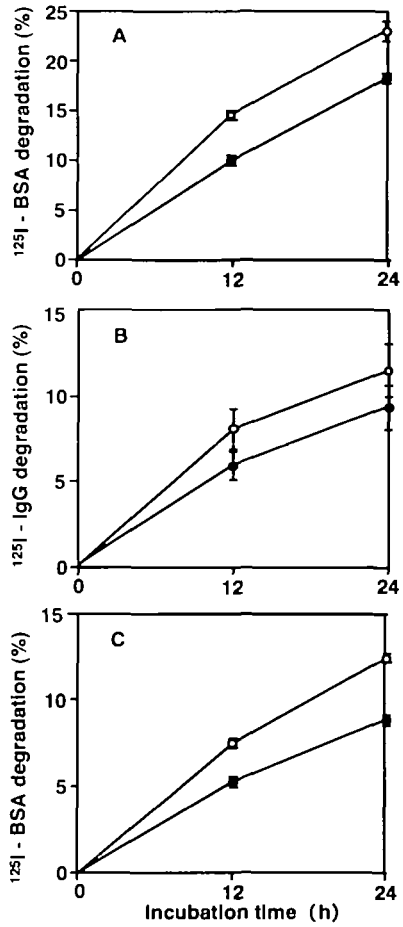

Fig. 7. **Time course of degradation of ^{125}I-unoxidized and oxidized BSA (A), ^{125}I-unoxidized and oxidized IgG (B), and ^{125}I-nonglycated and glycated BSA (C) by trypsin.** A mixture of (A) ^{125}I-unoxidized (●) and oxidized BSA (1 μg) (○), (B) ^{125}I-unoxidized (●) and oxidized IgG (1 μg) (○), or (C) ^{125}I-nonglycated (●) and glycated BSA (1 μg) (○), and trypsin (1 μg protein) in 105 μl of 10 mM sodium phosphate buffer (pH 8.0)/1 mM EDTA was incubated at 37°C for the indicated period. Protein degradation was examined by the release of trichloroacetic acid-soluble radioactivity. The data are the means of triplicate experiments.

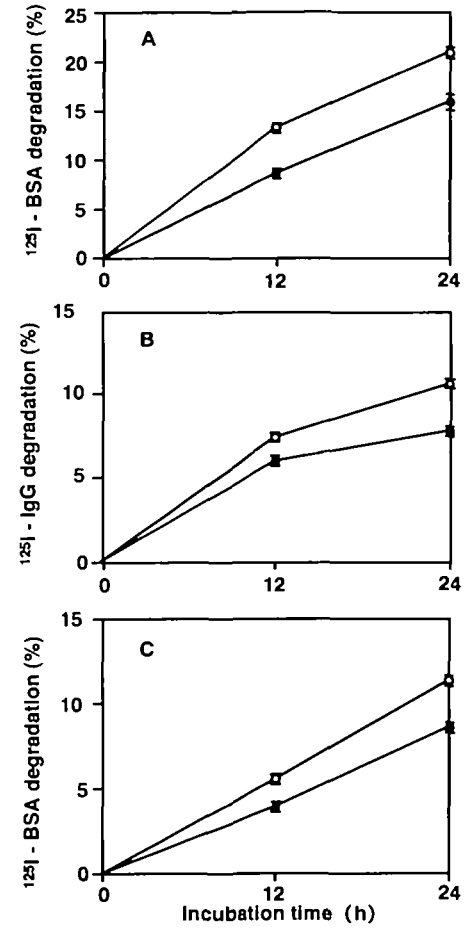

Fig. 8. **Time course of degradation of ^{125}I-unoxidized and oxidized BSA (A), ^{125}I-unoxidized and oxidized IgG (B), and ^{125}I-nonglycated and glycated BSA (C) by α-chymotrypsin.** A mixture of (A) ^{125}I-unoxidized (●) and oxidized BSA (1 μg) (○), (B) ^{125}I-unoxidized (●) and oxidized IgG (1 μg) (○), or (C) ^{125}I-nonglycated (●) and glycated BSA (1 μg) (○), and α-chymotrypsin (1 μg protein) in 105 μl of 10 mM sodium phosphate buffer (pH 8.0)/1 mM EDTA was incubated at 37°C for the indicated period. Protein degradation was examined by the release of trichloroacetic acid-soluble radioactivity. The data are the means of triplicate experiments.

readily degraded than the corresponding unmodified proteins throughout the incubation time. This result indicates that the 80-kDa protein preferentially degraded proteins damaged by oxidation or glycation.

Time courses of proteolysis by other representative serine proteases, trypsin (Fig. 7) and α-chymotrypsin (Fig. 8), of the ^{125}I-labeled modified and unmodified proteins showed that these serine proteases degraded oxidized BSA, oxidized IgG, and glycated BSA a little more effectively than the corresponding unmodified proteins. However, unlike the results for the 80-kDa protein (Fig. 6), the selectivity of these proteases for the modified proteins was very low. Thus, the observed selectivity of the 80-kDa protein for the modified proteins appears to be a unique characteristic of this protease.

Proteolytic activities of DEAE and native-PAGE fractions from native/[^{3}H]DFP-labeled cytosol toward ^{125}I-labeled oxidized BSA, oxidized IgG, and glycated BSA were effectively inhibited by DFP (Table III). The result confirms that the protease responsible for the degradation of the modified proteins was serine protease.

DISCUSSION

Our previous studies showed that oxidatively modified erythrocyte membrane proteins are degraded by an 80-kDa serine protease that is loosely bound to the membrane and may originate in erythrocyte cytosol (*6*). In the present study, the presence of an 80-kDa serine protease responsible for degradation of proteins damaged by oxidation and glycation in erythrocyte cytosol was elucidated. Physicochemical and immunochemical properties [*i.e.*, electrophoretic mobility in SDS- and native-PAGE (Fig. 1) and reactivity with antibody to cytosolic 80-kDa protein (Fig. 4)] of the membrane and the cytosolic 80-kDa protein indicated that they are identical. Furthermore, the observations that [^{3}H]DFP-labeled 80-kDa protein of cytosol

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

TABLE III. **Degradation of ^{125}I-labeled unmodified and modified proteins by DEAE fractions and native-PAGE fractions obtained from native/[^{3}H]DFP-labeled cytosol in the absence and presence of DFP.** A mixture of ^{125}I-labeled protein (1 μg) and DEAE fractions (4 μg protein) or native-PAGE fractions (9.5 μg protein) from native/[^{3}H]DFP-labeled cytosol in 105 μl of 10 mM sodium phosphate buffer (pH 8.0)/1 mM EDTA in the absence or presence of 2 mM DFP was incubated at 37°C for the indicated period. Degradation of BSA was measured by the release of trichloroacetic acid-soluble radioactivity. The data are the means±SD of triplicate experiments.

Preparation of 80-kDa protein (incubation h)	^{125}I-Protein degradation (%)			
	Unoxidized BSA		Oxidized BSA	
	− DFP	+ DFP	− DFP	+ DFP
DEAE fractions (120)	4.6±0.8	1.0±0.4	16.9±0.4	3.4±0.2
Native-PAGE fractions (96)	1.3±0.04	0.3±0.0	2.9±0.05	1.0±0.03
	Unoxidized IgG		Oxidized IgG	
	− DFP	+ DFP	− DFP	+ DFP
DEAE fractions (48)	1.1±0.5	0.4±0.2	6.7±0.5	1.2±0.5
	Nonglycated BSA		Glycated BSA	
	− DFP	+ DFP	− DFP	+ DFP
DEAE fractions (48)	1.1±0.2	0.6±0.1	7.5±0.8	1.4±0.2

bound to oxidized erythrocyte membranes more effectively than to unoxidized ones (Table I), and that a greater amount of 80-kDa protein is present in oxidized membranes than in unoxidized membranes as judged by the antibody binding (Fig. 4) indicated that the cytosolic enzyme became adherent to membranes when the cells were oxidized. This finding clearly explains our previous observation that the activity of the membrane-bound serine protease toward oxidized protein is greater in oxidized erythrocyte membranes than in unoxidized ones (*6*).

The protease was purified in a purity of 80% in a DFP-modified form by successive [^{3}H]DFP-labeling, CM-Sepahdex ion exchange chromatography, DEAE-cellulose ion exchange chromatography, and preparative native PAGE. The activity of DEAE fractions and native-PAGE fractions to degrade oxidized protein was effectively inhibited by DFP, indicating that the major activity is due to the 80-kDa serine protease. The DFP-uninhibitable protease activity that remains in these fractions may be ascribable to contamination by other protease species. In our previous work on the degradation of oxidized erythrocyte membrane proteins by the membrane-bound enzymes (*5*), the major contribution of a serine protease to the degradation of oxidized proteins was suggested by its effective inhibition by DFP. Therefore, the contribution of serine proteases appears to be important in the degradation of oxidized proteins in erythrocytes.

Erythrocyte membranes are known to be associated with several proteases, including calpain (*14*), cathepsin E (*15, 16*), acid proteinase (*17*), and multicatalytic proteinase (*18*), while erythrocyte cytosol contains proteases selective for oxidized hemoglobin or oxidized proteins (*2, 3*). The erythrocyte cytosolic proteases include 670-kDa (on gel filtration) and 21.5-35.7-kDa proteins (on SDS-PAGE under reducing conditions) (*2*) or 700-kDa (on gel filtration) and 23-32-kDa proteins (on SDS-PAGE under reducing conditions) (*3*). To our knowledge, the presence of an 80-kDa serine protease in erythrocyte membranes and cytosol has not hitherto been reported. Although the 80-kDa serine protease tended to lose its activity during the course of purification, as seen in the decreased activity of native-PAGE fractions as compared with DEAE fractions (Table III), the present method appears to be suitable for isolation of the enzyme protein for structural analysis. Using this purification method, the primary structure of this enzyme in erythrocyte cytosol is now under investigation.

Oxidized proteins are known to be susceptible to proteolytic degradation by proteases. The hydrophobicity of proteins is increased by oxidation, and the increase in degradability of oxidized proteins may be due to the increased hydrophobicity (*19-22*). Oxidation of proteins exposes hydrophobic residues (*21, 22*), and proteases such as the 80-kDa serine protease shown here may preferentially hydrolyze the surface hydrophobic residues of the oxidatively modified protein molecules.

The 80-kDa serine protease differed from the other serine proteases in the characteristics of its proteolytic activity. Unlike trypsin and α-chymotrypsin, the 80-kDa serine protease in erythrocyte cytosol was highly selective for the modified proteins. The protease may play the specific role of degrading proteins modified by oxidation and glycation. Because it was found in oxidized erythrocyte membranes (*5, 6*) and was highly adherent to oxidized erythrocyte membranes, it may play an important role in the degradation of oxidized proteins in the membranes.

Proteases that are specific to oxidatively damaged intracellular proteins have been found in erythrocyte and reticulocyte extracts (*2-4, 10, 23-26*), mitochondria (*27, 28*) and *Escherichia coli* extracts (*10, 29-31*). None of the proteases, however, has yet been identified. The 80-kDa serine protease found in erythrocyte membranes in the previous studies (*5, 6*) and in cytosol in the present study may be mainly present in cytosol and migrate to membranes when the cells are oxidized, and thus participate in the degradation of oxidized membrane proteins.

REFERENCES

1. Davies, K.J.A. (1986) Intracellular proteolytic systems may function as secondary antioxidant defenses: An hypothesis. *Free Rad. Biol. Med.* **2**, 155-173
2. Pacifici, R.E., Salo, D.C., and Davies, K.J.A. (1989) Macroxyproteinase (M.O.P.): A 670 kDa proteinase complex that degrades oxidatively denatured proteins in red blood cells. *Free Rad. Biol. Med.* **7**, 521-536
3. Sacchetta, P., Battista, P., Santarone, S., and Cola, D.D. (1990) Purification of human erythrocyte proteolytic enzyme responsible for degradation of oxidant-damaged hemoglobin. Evidence for identifying as a member of the multicatalytic proteinase family. *Biochim. Biophys. Acta* **1037**, 337-343
4. Fagan, J.M. and Waxman, L. (1991) Purification of a protease in red blood cells that degrades oxidatively damaged haemoglobin. *Biochem. J.* **277**, 779-786
5. Beppu, M., Inoue, M., Ishikawa, T., and Kikugawa, K. (1994) Presence of membrane-bound proteinases that preferentially degrade oxidatively damaged erythrocyte membrane proteins as secondary antioxidant defense. *Biochim. Biophys. Acta* **1196**, 81-87
6. Fujino, T., Ishikawa, T., Inoue, M., Beppu, M., and Kikugawa, K. (1998) Characterization of membrane-bound serine protease related to degradation of oxidatively damaged erythrocyte membrane proteins. *Biochim. Biophys. Acta* **1374**, 47-55
7. Beppu, M., Mizukami, A., Nagoya, M., and Kikugawa, K. (1990) Binding of anti-band 3 autoantibody to oxidatively damaged

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

erythrocytes. *J. Biol. Chem.* **265**, 3226-3233
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275
9. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
10. Davies, K.J.A. (1987) Protein damage and degradation by oxygen radicals. *J. Biol. Chem.* **262**, 9895-9901
11. Johnstone, A. and Thorpe, R., eds. (1982) *Immunochemistry in Practice*, p. 2, Blackwell Scientific Publications, Boston
12. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354
13. Bolton, A.E. and Hunter, W.M. (1973) The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J.* **133**, 529-538
14. Murakami, T., Hatanaka, M., and Murachi, T. (1981) The cytosol of human erythrocytes contains a highly Ca^{2+}-sensitive thiol protease (calpain I) and its specific inhibitor protein (calpastatin). *J. Biochem.* **90**, 1809-1816
15. Yamamoto, K., Takeda, M., Yamamoto, H., Tatsumi, M., and Kato, Y. (1985) Human erythrocyte membrane acid proteinase (EMAP): Sidendness and relation to cathepsin D. *J. Biochem.* **97**, 821-830
16. Yamamoto, K. and Marchesi, V.T. (1984) Purification and characterization of acid proteinase from human erythrocyte membranes. *Biochim. Biophys. Acta* **790**, 208-218
17. Pontremoli, S., Salamino, F., Sparatore, B., Melloni, E., Morelli, A., Benatti, U., and Flora, A.D. (1979) Isolation and partial characterization of three acidic proteinases in erythrocyte membranes. *Biochem. J.* **181**, 559-568
18. Kinoshita, M., Hanakubo, T., Fukui, I., Murachi, T., and Toyohara, H. (1990) Significant amount of multicatalytic proteinase identified on membrane from human erythrocyte. *J. Biochem.* **107**, 440-444
19. Davies, K.J.A., Delsignore, M.E., and Lin, S.W. (1987) Protein damage and degradation by oxygen radicals II. Modification of amino acids. *J. Biol. Chem.* **262**, 9902-9907
20. Davies, K.J.A. and Delsignore, M.E. (1987) Protein damage and degradation by oxygen radicals III. Modification of secondary and tertiary structure. *J. Biol. Chem.* **262**, 9908-9913
21. Cervera, J. and Levine, R.T. (1988) Modulation of the hydrophobicity of glutamine synthetase by mixed-function oxidation. *FASEB. J.* **2**, 2591-2595
22. Pacifici, R.E., Kono, Y., and Davies, K.J.A. (1993) Hydrophobicity as the signal for selective degradation of hydroxy radical-modified hemoglobin by the multicatalytic proteinase comples, proteasome. *J. Biol. Chem.* **268**, 15405-15411
23. Goldberg, A.L. and Boches, F.S. (1982) Oxidized proteins in erythrocytes are rapidly degraded by the adenosine triphosphate-dependent proteolytic system. *Science* **215**, 1107-1109
24. Fagan, J.W.M., Waxman, L., and Goldberg, A.L. (1986) Red blood cells contain a pathway for the degradation of oxidant-damaged hemoglobin that does not require ATP or ubiquitin. *J. Biol. Chem.* **261**, 5705-5713
25. Davies, K.J.A. and Goldberg, A.L. (1987) Oxygen radicals stimulate intracelluar proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. *J. Biol. Chem.* **262**, 8220-8226
26. Davies, K.J.A. and Goldberg, A.L. (1987) Proteins damaged by oxygen redicals are rapidly degraded in extracts of red blood cells. *J. Biol. Chem.* **262**, 8227-8234
27. Dean, R.T. and Pollak, J.K. (1985) Endogenous free radical generation may influence proteolysis in mitochondria. *Biochem. Biophys. Res. Commun.* **126**, 1082-1089
28. Wolff, S.P., Garner, A., and Dean, R.T. (1986) Free radicals, lipids and protein degradation. *Trends Biol. Sci.* **11**, 27-31
29. Levine, R.L., Oliver, C.N., Fulks, R.M., and Stadtman, E.R. (1981) Turnover of bacterial glutamine synthetase: Oxidative inactivation precedes proteolysis. *Proc. Natl. Acad. Sci. USA* **78**, 2120-2124
30. Davies, K.J.A. and Lin, S.W. (1988) Degradation of oxidatively denatured proteins in *Escherichia coli*. *Free Rad. Biol. Med.* **5**, 215-223
31. Davies, K.J.A. and Lin, S.W. (1988) Oxidatively denatured proteins are degraded by an ATP-independent proteolytic pathway in *Escherichia coli*. *Free Rad. Biol. Med.* **5**, 225-236

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012